PRIMARY STRUCTURE OF HEMOGLOBINS FROM TROUT *(SALMO IRIDEUS)*. PARTIAL DETERMINATION OF AMINO ACID SEQUENCE OF HB TROUT IV

F. BOSSA, D. BARRA, M. COLETTA, F. MARTINI, A. LIVERZANI, R. PETRUZZELLI, J. BONAVENTURA* and M. BRUNORI

Centro di Biologia Molecolare del C.N.R. e Istituti di Chimica e Chimica Biologica, Facoltà di Medicina, Università di Roma, Italy

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1. Introduction

Recent work on the properties of the hemoglobin components from the trout *Salmo irideus* has brought to focus a number of interesting features bearing on the problem of molecular adaptation to physiological requirements [1,2]. Hb trout IV is characterized by a strong dependence of both ligand affinity and cooperativity on pH and organic phosphates; this is the so-called Root effect, which is related with delivery of O_2 to the swim bladder in teleost fishes. Hb trout I lacks completely proton and phosphate linked heterotropic effects, although ligand binding is strongly cooperative, it is supposed to be an important O_2 delivery-system present in hyperactive fish, which may undergo serious O_2 debts under conditions of acidosis. Thus the presence of multiple hemoglobin components with different functional properties can be given a rational interpretation on the basis of multiple physiological demands.

Any attempt to interpret the functional properties of these hemoglobins in terms of structural features demands, as a necessary prerequisite, the knowledge of their primary structure. Therefore a major goal of the work on the structure–function relationships in the hemoglobin components from trout is the determination of their amino acid sequence. In a previous communication [3] we reported information on the C- and N-terminal regions of both Hb trout I and Hb trout IV, and commented on the implications of these findings for the function of the two proteins. In this paper we present part of the amino acid sequence of both the α and β chains of Hb trout IV. The results, although still incomplete, provide an opportunity to attempt a number of interesting comparisons with mammalian Hbs as well as with other fish Hbs. In addition, they allow one to draw significant conclusions on the role of several 'Key-residues' in determining the characteristic functional properties of these molecules.

2. Experimental

Hb trout IV was separated from other hemoglobin components according to a procedure reported elsewhere [4] and globin was prepared by the acid–acetone method [5].

All chromatographic attempts to separate the chains without blocking the -SH groups failed, while good separation was obtained starting from globin carboxymethylated according to the usual procedure [6]. This material (about 300 mg) was adsorbed onto a column of Whatman CM 32 (2.5 × 24 cm) equilibrated with 30 mM sodium phosphate buffer, pH 7.6, containing 8 M urea and 75 mM 2-mercaptoethanol. The chains were eluted using a linear gradient with 250 ml of starting buffer in the mixing chamber and 250 ml of 75 mM sodium phosphate buffer, pH 6.7, containing 8 M-urea and 75 mM 2-mercaptoethanol in the reservoir. Homogeneity of the chromatographic components was checked by disc electrophoresis on polyacrylamide in 10 M urea.

* Present address: Duke University, Marine Laboratory, Beaufort, N. C. 28516, USA.

Aliquots of the isolated α or β chains were subjected to tryptic or chymotryptic digestion [7]. The soluble tryptic or chymotryptic peptides were separated according to a common general scheme including (i) initial Sephadex G-25 gel filtration of the digest, (ii) subsequent fractionation by ion-exchange chromatography and (iii) final purification with paper chromatography and/or high voltage paper electrophoresis. Details of these procedures and of the experimental methods used to check purity of fractions, to determine amino acid analysis, N- and C-terminal residues and sequence of peptides were already reported [8].

3. Results and discussion

About 70% of the sequence of Hb trout IV has been deduced from an investigation of the soluble peptides released by tryptic digestion of the α chain, and by tryptic and chymotryptic digestion of the β chain. Many of these peptides could be unambiguously aligned with other hemoglobins on the basis of homology considerations. In particular, it has been possible to construct a 61 residues N-terminal sequence for the α chain and a 85 residues (with a gap of three residues at positions 43–45) N-terminal sequence for the β chain. These sequences are reported in fig.1 and 2, compared with the corresponding sequences of human hemoglobin.

Details on the purification procedure and on the evidence for the sequence data of each individual peptide and for the overlaps will be presented when determination of the complete structure of the chains will be accomplished.

As far as it concerns fish hemoglobins the sequences of two more α chains, namely those of carp [9] and of *Catostomus clarkii* [10] hemoglobin, are known. The N-terminal portion of the latter is also reported in fig.1, since the corresponding hemoglobin seems strictly related to Hb trout IV from the functional point of view [11]. Extensive sequence data for β chains of fish hemoglobins other than Hb trout IV are presently not available.

As reported before [3], the last two C-terminal residues of both chains in Hb trout IV are identical to

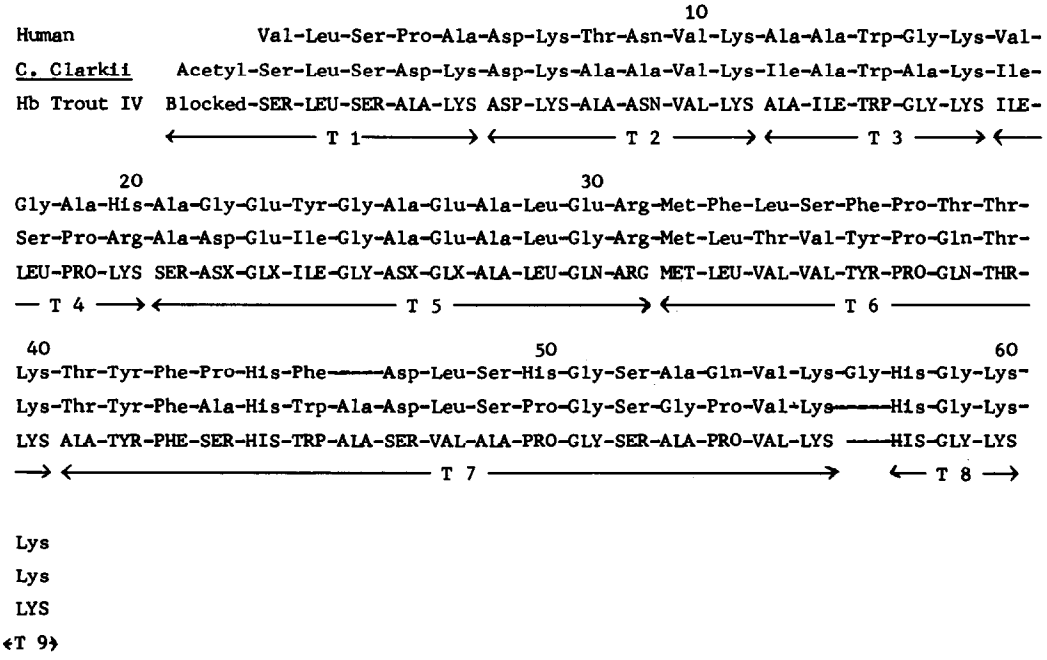

Fig.1. Comparison of the N-terminal amino acid sequence of HbA (human), Hb *Catostomus clarkii* and Hb trout IV α chains. Tryptic peptides (T) are represented by arrows and numbered from the N-terminus of the molecule.

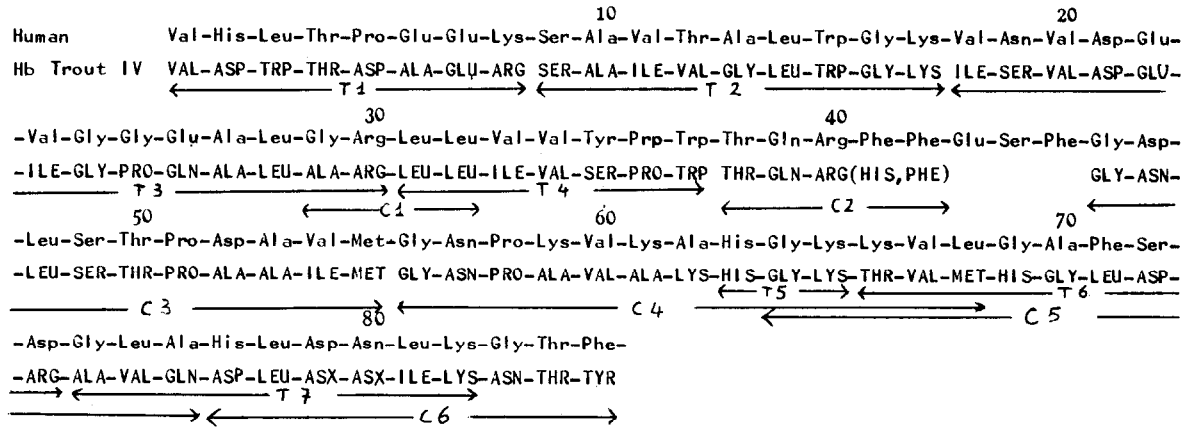

Fig.2. Comparison of the N-terminal amino acid sequence of HbA (human) and Hb Trout IV β chains. Peptides are represented by arrows and are identified by a capital letter (T = tryptic and C = chymotryptic). Each peptide is numbered from the N-terminus of the molecule.

those found in HbA (i.e. – Tyr – Arg for α and – Tyr – His for β). Moreover the proximal and distal histidines are present in both chains, (even though former ones are not shown in figs.1 and 2). The α-amino group of the α chain is acetylated in carp and *Catostomus clarkii* hemoglobins and is blocked also in Hb trout IV, but identification of the blocking group has not been attempted.

Fig.3 reports the molecular contacts in the $\alpha_1\beta_2$ (or $\alpha_2\beta_1$) interface for human Hb [12]; the corresponding residues for Hb trout IV are also given. Some of these residues are in sections of the sequence not reported in figs.1 and 2, but already available.

Very many of the amino acid residues in the interface are identical both in the liganded and unliganded

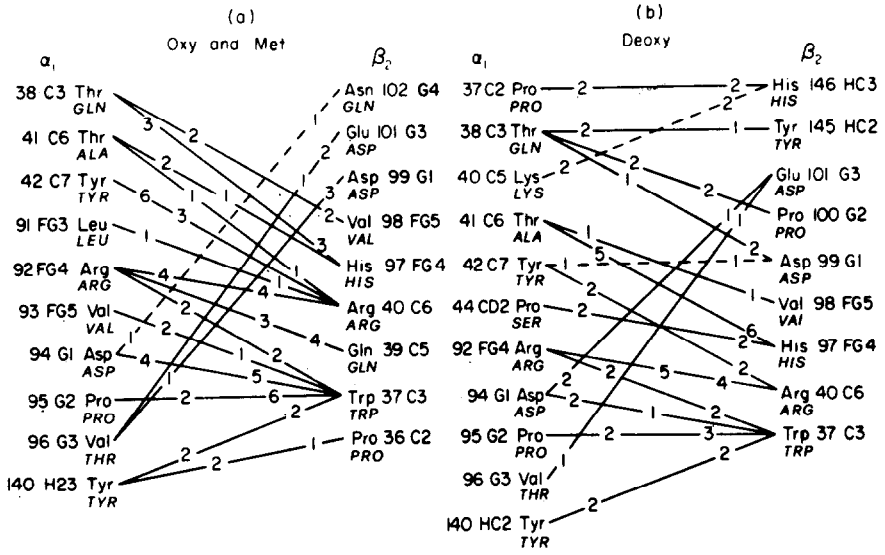

Fig.3. The $\alpha_1\beta_2$ contacts in oxy- and deoxy-hemoglobin in both HbA and Hb Trout IV. Residues in HbA are represented as given by Perutz ([12], modified). Below each residue of HbA the corresponding residues of Hb trout IV are reported (in capital letters).

derivatives. More substitutions are observed in the α chains (3 in oxy and 4 in deoxy) than in the β chains (1 in oxy and 1 in deoxy); in the latter case they are conservative i.e.: 102 (β) G4 Asn → Gln or 101 (β) G3 Glu → Asp).

Among the residues in this interface between α and β chains, Trp-37 (β) C3 and Tyr-42 (α) C7 have acquired significance as spectral probes for the conformational transition in human Hb. Evidence has been accumulated that the features of the difference spectrum observed in the ultraviolet region (between 285 and 295 nm) reflect modifications in the environment of these two amino acid residues, and thus structural changes in the $\alpha_1\beta_2$ interface [13]. A similar spectral change has been observed in carbonmonoxide Hb trout IV on going from pH 8 to pH 6, i.e. over the pH region where the Root effect is operative ([14], M. F. Perutz, unpublished data). This has been taken as one of the indications that protons can induce in ligand bound Hb trout IV a quaternary transition R → T. In turn, this result also implies, by analogy with human hemoglobin, that lowering pH induces, in ligand bound Hb trout IV, a structural change at the level of the $\alpha_1\beta_2$ interface similar to that observed in human hemoglobin. This is supported by the fact that all the residues in the $\alpha_1\beta_2$ interface which interact with both Trp-37 (β) C3 and Tyr-42 (α) C7 are maintained in Hb trout IV (see fig.3).

It is particularly significant that both Tyr-42 (α) C7 and Asp-99 (β) G1, which contribute a critical H-bond in *deoxy* Hb [12], are found also in Hb trout IV. The H-bond in *oxy* between Asp-94 (α) G1 and Asn-102 (β) G4, which in Hb trout IV is substituted by Gln, should also be present, although it cannot be stated with certainty.

Among the changes, those involving His-97 (β) FG4 may be significant. This residue makes the following molecular contacts (4 Å or less):

oxy { 38 (α) C3 Thr → Gln ; 41 (α) C6 Thr → Ala

deoxy { 41 (α) C6 Thr → Ala ; 44 (α) CD2Pro → Ser

In deoxy Hb Thr-38 (α) C3 is also in molecular contact with Asp-99 (β) G1, which makes a H-bond with Tyr-42 (α) C7. As reported above, this is a critical H-bond for stabilising the deoxy structure, and arguments for the critical nature of this H-bond have been given by Perutz [15]. No substitution of Thr-38 (α) C3 have been found as yet in abnormal human hemoglobins or other hemoglobins [10]. On the other hand fish hemoglobin examined up to now all present a glutamine at this position [9,10].

In addition it is significant that also the other residues interacting with His-97 (β) FG4 are substituted in Hb trout IV. Thus it seems clear that substitution of Thr 41 (α) C6 by Ala, and Pro-44 (α) CD2 by Ser may result in alterations of the molecular interactions involving His-97 (β) FG4 in the $\alpha_1\beta_2$ interface. The latter substitution, for example, may be related to the very small value of the tetramer–dimer dissociation constant in Hb trout IV [1].

It is not possible at this stage to provide any water-tight argument for the critical role of these substitutions. However with the information presently available it seems reasonable to propose, as a working hypothesis, that these substitutions will play a significant role in modifying the relative stability of the quaternary states of oxy and deoxy Hb trout IV, and therefore in determining the Root effect in fish hemoglobins.

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References

[1] Brunori, M., Bonaventura, J., Bonaventura, C., Giardina, B., Bossa, F. and Antonini, E. (1973) Mol. Cell. Biochem. 1, 189–196.
[2] Brunori, M. (1975) Curr. Topics in Cell. Regul. 9, 1–39.
[3] Barra, D., Bossa, F., Bonaventura, J. and Brunori, M. (1973) FEBS Lett. 35, 151–154.
[4] Binotti, I., Giovenco, S., Giardina, B., Antonini, E., Brunori, M. and Wyman, J. (1971) Arch. Biochem. Biophys. 142, 274–280.
[5] Rossi Fanelli, A., Antonini, E. and Caputo, A. (1958) Biochem. Biophys. Acta 30, 608–615.

[6] Crestfield, A. M., Moore, S. and Stein, W. H. (1963) J. Biol. Chem. 238, 622–627.
[7] Smith, D. G. (1967) in: Methods in Enzymology (Hirs, C. H. W. ed.) vol. 17, pp. 214–231, Academic Press, New York.
[8] Bossa, F., Barra, D., Carloni, M., Fasella, P., Riva, F., Doonan, S., Doonan, H. J., Hanford, R., Vernon, C. A. and Walker, J. M. (1973) Biochem. J. 133, 805–819.
[9] Hilse, K. and Braunitzer, G. (1968) Hoppe-Seyler's Z. Physiol. Chem. 349, 433–450.
[10] Powers, D. A. and Edmunson, A. B. (1972) J. Biol. Chem. 247, 6694–6707.
[11] Powers, D. A. and Edmunson, A. B. (1972) J. Biol. Chem. 247, 6686–6693.
[12] Bolton, W. and Perutz, M. F. (1970) Nature 228, 551–552.
[13] Perutz, M. F., Ladner, J. E., Simon, R. S. and Chien Ho (1974) Biochemistry 13, 2163–2173.
[14] Giardina, B., Ascoli, F. and Brunori, M. (1975) Nature 256, 761–762.
[15] Perutz, M. F. and Teneyek, L. F. (1971) Cold Spring Harbor Symp. Quant. Biol. 36, 295–310.